A PURIFICATION OF VENOM PHOSPHODIESTERASE*

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For purposes of analysis of polynucleotides, it is desirable to have a phosphodiesterase, substantially free of 5-nucleotidase or other phosphatase activity. The presence of a phosphodiesterase in a wide variety of snake venoms was demonstrated by Gulland and Jackson (1). These venoms were found also to contain a potent 5-nucleotidase, but were free of alkaline phosphatase activity. Hurst and Butler (2) found that certain samples of Russell's viper venom were nearly free of 5-nucleotidase activity, while retaining potent phosphodiesterase action. By a chromatographic procedure, involving the use of cellulose columns, they were able to reduce the 5-nucleotidase activity of rattlesnake venom, relative to its phosphodiesterase activity, and to obtain fractions nearly comparable to the viper venom.

For certain purposes, however, such as the determination of the ratio of sequential isomers in mixed isomeric dinucleotides, the preparations obtained from rattlesnake venom by the chromatographic procedure of Hurst and Butler are not sufficiently free of 5-nucleotidase action. Therefore, a simple acetone fractionation has been developed, which yields a potent phosphodiesterase, substantially free of 5-nucleotidase activity, from rattlesnake venom. With this preparation, it has been possible to obtain quantitative degradation of di- and trinucleotides to mononucleotides, and to degrade a complete desoxyribonuclease digest to mononucleotides with the conversion of only 1 per cent of the digest to nucleosides.

Materials and Methods

Materials—The rattlesnake venom employed has been that of the eastern diamond-back rattlesnake (*Crotalus adamanteus*) obtained in lyophilized form from Ross Allen's Reptile Institute, Silver Springs, Florida.

Adenosine-5-PO_4 was obtained from the Sigma Chemical Company.

Ca[bis(*p*-nitrophenyl)phosphate]$_2$ was prepared from tris(*p*-nitrophenyl)-phosphate according to the method of Yoshida (3). The tris(*p*-nitrophenyl)phosphate was prepared according to Rapp[1] (4).

Assay for 5-Nucleotidase Activity—To 0.4 ml. of enzyme solution, ap-

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[1] It is important to use a considerable (10-fold) excess of fuming nitric acid in the nitration of the triphenyl phosphate according to Rapp's procedure.

propriately diluted, add 0.4 ml. of adenosine-5-PO_4 (450 γ per ml.), 0.2 ml. of 0.3 M $MgCl_2$, and 1.0 ml. of 0.1 M glycine buffer, pH 8.8. After a 3 hour incubation at 37°, 1.0 ml. of 0.5 M acetate buffer, pH 4.0, is added. Inorganic phosphate is then determined according to the method of Lowry and Lopez (5). The reduced phosphomolybdate was assayed by the absorption at 800 mμ, measured in the Beckman spectrophotometer.

Assay for Phosphodiesterase Activity—To 0.4 ml. of enzyme, add 0.6 ml. of 0.1 M glycine buffer, pH 8.8, 0.2 ml. of 0.3 M magnesium acetate, and 1.0 ml. of 0.001 M Ca[bis(*p*-nitrophenyl)phosphate]$_2$. After incubation for 3 hours at 37°, a 1 ml. aliquot is removed, added to 2 ml. of H_2O, and the absorption at 440 mμ read in the Beckman instrument (for complete digestion, $D_{440} = 2.0$).

Purification of Venom Phosphodiesterase—1 gm. of rattlesnake venom is suspended in 100 ml. of H_2O at room temperature. Any insoluble material is removed by centrifugation and discarded. To the supernatant solution are added 67 ml. of reagent acetone. The mixture is allowed to stand for 1 hour in the cold room, after which it is centrifuged for 1 hour at 10,000 r.p.m. in the Servall centrifuge. The precipitate is discarded. To the supernatant solution are added 133 ml. of reagent acetone. The mixture is allowed to stand overnight in the cold room, after which it is centrifuged for 1 hour at the full speed of the Servall centrifuge (16,000 r.p.m.). The supernatant solution is discarded.

The precipitate is suspended in 100 ml. of H_2O and any insoluble matter is removed by centrifugation. The 40 and 67 per cent acetone cycle is then repeated. The final sediment is dissolved in 32 ml. of H_2O and any insoluble matter centrifuged.

If desired, further purification of the phosphodiesterase preparation may be achieved by selective thermal inactivation. The preparation is divided into twenty 1.5 ml. portions and incubated for 40 minutes in a water bath at just 65°.

Results

After the second acetone fractionation, the redissolved preparation is 18 to 20 per cent as active as the original venom solution as regards phosphodiesterase activity, and 0.02 to 0.03 per cent as active as regards 5-nucleotidase activity. This preparation is adequate for quantitative degradation of di- and trinucleotides to mononucleotides. The subsequent thermal inactivation destroys 60 per cent of the diesterase activity, but reduces the 5-nucleotidase activity below a detectable level in the test system employed.

With the thermally treated preparation, it is possible to obtain a nearly quantitative degradation of a desoxyribonuclease digest of desoxyribo-

nucleic acid (DNA) to mononucleotides. 2 mg. of a highly polymerized, incompletely dried preparation of calf thymus DNA (6) (containing 150 γ of P) were dissolved in 0.5 ml. of 0.5 M acetate buffer, pH 6.5. To this were added 0.4 ml. of a solution of crystalline desoxyribonuclease (Worthington Biochemical Sales Company) containing 100 γ per ml., 0.2 ml. of 0.3 M magnesium acetate, and 0.6 ml. of H_2O. After incubation for 48 hours at 37°, under hexane, during which time the ultraviolet absorption at 260 mμ increased by 48 per cent, 1.0 ml. of 0.2 M NH_4OH-NH_4 acetate buffer, pH 9.2, was added, plus 0.25 ml. of the diesterase preparation. After further incubation for 6 hours at 37°, during which time the ultraviolet absorption at 260 mμ increased a further 26 per cent, the digest was fractionated into mononucleotides by ion exchange chromatography on Dowex 1, essentially according to the procedure previously described[2] (7).

TABLE I

Molar Proportions of Mononucleotides in Calf Thymus DNA

	5-Methyl-cytidylic	Cytidylic	Thymi-dylic	Adenylic	Guanylic
Diesterase digest	0.067	0.83	1.09	1.15	0.87
Data of Wyatt (8)	0.052	0.85	1.11	1.13	0.86

A column 3 mm. in diameter and 10 cm. in height was employed, with a flow rate of 2.3 ml. per hour.

Only 1 per cent of the digest appeared as nucleosides, the remainder being accounted for as mononucleotides. The molar proportions of the mononucleotides obtained are in generally good agreement with those obtained by other workers (Table I).

SUMMARY

1. A procedure is described for the purification of rattlesnake venom phosphodiesterase from 5-nucleotidase activity by acetone fractionation and selective thermal inactivation.

2. With the purified phosphodiesterase preparation, it is possible to obtain substantially quantitative degradation of desoxyribonuclease digests to mononucleotides.

[2] One important modification to the published procedure is the use of 0.03 M acetate buffer, pH 4.3, to elute the desoxy-5-methylcytidylic and desoxycytidylic acids. The lower acetate concentration permits complete resolution of these two nucleotides.

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